A FERROCENE BASED GLUCOSE BIOSENSOR WITH AN EXTENDED LINEAR MEASURING RANGE

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ABSTRACT

The work described in this research is centred on the performance of ferrocene as a mediator to replace oxygen in the oxidation of glucose. Three immobilization methods were studied to minimize the leaking of the mediator. Glucose oxidase (GOD) and ferrocene mediator were immobilized in cross-linked poly(vinyl alcohol) (CLPVA), composite sol-gel-silica (SGS)/CLPVA/nafion and layer-by-layer (multilayer) covalent attachment. The biosensor response was evaluated amperometrically at 0.363V (vs. Ag/AgCl). The results showed that SGS/CLPVA/nafion membrane demonstrated the highest ability in retaining the enzyme and mediator whilst CLPVA membranes showed the poorest ability. The low current response with high Michaelis-Menten constant (K_m^{app}) and slow response time was observed in CLPVA membrane. Relative to that, SGS/CLPVA/nafion membrane and multilayered membrane were able to achieve larger current responses with lower K_m^{app} at faster response time. CLPVA was able to retain only 10% of the initial current whilst SGS/CLPVA/nafion and multilayered membranes were able to retain about 83% and 76% respectively of the initial current after 2 months of storage. All membranes showed good sensitivity with good stability except the CLPVA membranes. The K_m^{app} value for multilayered membrane was increased up to 28.68mM after the addition of nafion to the membrane. With such a high K_m^{app}, multilayered-nafion membrane can be applied as the enzymatic layer for a glucose biosensor since the value nearly encompasses the relevant concentration of glucose in blood which is from 1mM to 30mM. Subsequently, three types of external membranes were attached to the multilayered-nafion membranes to extend the linearity of sensor response and also to protect the biosensor. The external layers were cross-linked poly(2-hydroxyethyl methacrylate) (pHEMA), cellulose acetate incorporated poly(2-hydroxyethyl methacrylate) (CA-pHEMA) and cellulose acetate incorporated poly(ethylene glycol) (CA-PEG). Among these three types outer membranes, multilayered-nafion membranes with cross-linked pHEMA demonstrated acceptable K_m^{app}, which was around 40.58mM with high sensitivity and fast response time towards the glucose. Overall, the ferrocene based glucose biosensor showed very promising performances.

ABSTRAK

Penyelidikan ini tertumpu kepada kajian prestasi pengantara ferosena dalam menggantikan oksigen dalam proses pengoksidaan glukosa. Tiga kaedah penyekatgerakan dikaji untuk mengurangkan kebocoran pengantara. Glukosa oksidase (GOD) dan pengantara ferosena disekatgerakan dalam poli(vinil alkohol) tersambung-silang (CLPVA), komposit sol-gel-silika (SGS)/CLPVA/nafion dan pelekatan kovalen lapisan demi lapisan (multilapisan). Tindakbalas biosensor ditentukan secara amperometrik pada 0.363V (lwn. Ag/AgCl). Keputusan menunjukkan membran SGS/CLPVA/nafion mempunyai kemampuan yang tertinggi dalam menahan enzim dan pengantara sementara membran CLPVA menunjukkan kemampuan yang paling lemah. Tindakbalas arus yang rendah dengan pemalar Michaelis-Menten (K_m^{app}) yang besar dan masa tindakbalas yang perlahan adalah diperhatikan pada membran CLPVA. Relatif kepada pencapaian tersebut, membran SGS/CLPVA/nafion dan membran multilapisan pula mampu mencapai tindakbalas arus yang lebih besar dengan K_m^{app} yang lebih rendah pada masa tindakbalas yang lebih cepat. Membran CLPVA hanya mampu mengekalkan 10% daripada nilai awal arus sementara membran SGS/CLPVA/nafion dan multilapisan masing-masing mampu mengekalkan sebanyak 83% dan 76% daripada nilai awal arus selepas penyimpanan selama 2 bulan. Semua membran menunjukkan sensitiviti yang baik dengan kestabilan yang baik kecuali membran CLPVA. Nilai K_m^{app} untuk membran multilapisan meningkat sehingga 28.68mM selepas penambahan nafion pada membran tersebut. Dengan nilai K_m^{app} yang tinggi, membran multilapisan-nafion boleh diaplikasikan sebagai lapisan enzimatik biosensor glukosa memandangkan nilainya hampir mencapai kepekatan relevan glukosa dalam darah iaitu antara 1mM hingga 30mM. Seterusnya, tiga jenis membran luaran ditambah kepada membran multilapisan-nafion untuk melanjutkan kelinearan tindakbalas sensor dan juga untuk melindungi biosensor. Lapisan-lapisan luaran tersebut adalah poli(2-hidroksi etil metakrilat) (pHEMA) tersambung-silang, selulosa asetat dengan poli(2-hidroksi etil metakrilat) (CA-pHEMA) dan selulosa asetat dengan poli(etilen glikol) (CA-PEG). Antara ketiga-tiga membran luaran ini, pHEMA tersambung-silang mempamerkan K_m^{app} yang sesuai iaitu 40.58mM dengan sensitiviti yang tinggi dan masa tindakbalas yang pantas terhadap glukosa. Keseluruhannya, biosensor berdasarkan ferosena telah menunjukkan prestasi yang memberangsangkan.

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Research Background

Diabetes mellitus is a disease in which the body cannot produce sufficient insulin in their pancreas to adequately control the level of glucose in their blood (Eggins, 1996). Therefore, people with diabetes have to maintain a balance between carbohydrate intake and insulin production to enable their metabolism to run in a stable manner. Henning and Cunningham (1998) have used the simplest model of how the body regulates glucose. Elevation of glucose in the blood by adsorption of glucose from food will stimulate the islet cells in the pancreas to secrete the hormone insulin. Insulin acts on the cells of the body to take up the glucose. If the body feels a shortage, insulin production is slowed and the liver releases glucose that is stored as glycogen.

There are two type of diabetes (Henning and Cunningham, 1998). Type 1 diabetes is sometimes referred to as *juvenile diabetes* or *insulin dependent diabetes mellitus* (IDDM) and usually strikes children and young adults. The insulin producing islet cells in the pancreas are destroyed by the diabetic's own immune system. These type 1 diabetics usually lose all insulin-producing capabilities and

must inject themselves with insulin before each meal to allow their bodies to utilize glucose from the food. Meanwhile, type 2 diabetes is referred as *non insulin dependent diabetes mellitus* (NIDDM) and usually is suffered by older people. Type 2 diabetics can usually increase their glucose regulation by losing weight and are initially treated by diet control and with drugs that help the body metabolize glucose. Type 2 diabetics over time may need to start using insulin injections to maintain glucose regulation.

Glucose is important because of its involvement in human metabolic processes (Eggins, 1996). As the primary source of energy for the brain as well as all other cells in the body, the consequences of poor glucose regulation are, long term damage to organs from too much glucose (hyperglycemia) and coma or death caused by too little glucose reaching the brain (hypoglycemia) (Henning and Cunningham, 1998). The insulin dose must be adjusted to minimize hyperglycemia while avoiding serious hypoglycemia (Buerk, 1993). The Diabetes Control and Complications Trials Research Group (DCCT) (1996) reported that severe hypoglycemia is particularly problematic because of its potential influence on the integrity of the central nervous system that affects activities of daily living and may also lead to clinically significant brain damage if untreated.

The detection of glucose has attracted a high degree of interest due to its biological importance (Liu *et al.*, 2004). About half of biosensor research papers published is based on glucose detection (Eggins, 2002). A biosensor is a sensor that is based on the use of biological material for its sensing function. Generally, glucose biosensors are based on the fact that the enzyme glucose oxidase (GOD) catalyzes the oxidation of glucose to gluconic acid (Eggins, 1996). Even though expensive, GOD enzymes are stable over a long period of time and are also highly selective for glucose in the presence of other sugars in blood solution (Eggins, 2002). With a combination of advanced electrochemical technique and high substrate specificity, amperometric glucose biosensor represents the most successful commercial biosensor development with rapid and accurate diagnosis (Sulak *et al.*, 2005).

Electrochemical sensing of glucose has been classified according to three generations. In the first generation, GOD enzyme catalyzes the oxidation of glucose to gluconic acid with the production of hydrogen peroxide. However, by using an artificial electron acceptor or mediator to replace the natural acceptor oxygen, this second generation approach is preferable as it is capable of overcoming tissue oxygen dependence problem suffered by first generation glucose biosensors. In addition, the oxidation of the reduced mediator occurs at low potential thus reducing the sensitivity of the sensor to interfering substances (Cardosi and Turner, 1991; Reynolds *et al.*, 1992; Eggins, 1996; Chaubey and Malhotra, 2002). The third generation biosensors are based on directly coupled enzyme electrode using conducting salt electrodes (Eggins, 1996).

Even though the use of mediator for a glucose biosensor has a lot of advantages, however, the loss of mediator is an important issue because of the inherent toxic effect of mediators. This is especially true for implantable sensors. Therefore, the performance of a mediated glucose biosensor depends partly on the methods of tethering a mediator to an enzymatic membrane. Other restraining factor in glucose biosensor is limited linearity of the biosensor response to higher glucose concentrations. Since glucose level in diabetic blood can reach up to 30mM (Cass *et al.*, 1984; Mascini and Moscone, 1991), limited linearity of the calibration curve will pose a problem as direct measurement without any dilution is impossible.

Therefore, in order to develop a stable mediated glucose sensor, three different immobilization methods have been investigated to minimize leaking of mediator as well as the enzyme and to extend the linearity of glucose sensor response. Performances of the three types of membranes were compared and the best method was selected for further studies with various types of outer membranes to further extend the linear range to higher glucose concentrations by limiting the flux of glucose to the enzyme layer. Amongst the analyses that have been conducted were current response, response time, enzyme kinetics, membranes stability, and leakage detection of both ferrocene and also glucose oxidase enzyme. All electrochemical studies were done by attaching the membranes to platinum electrode.

In the first method, the enzyme and the poly(allylamine) ferrocene (PAA-Fc) were immobilized in cross-linked poly(vinyl alcohol) (CLPVA) with the addition of bovine serum albumin (BSA) as protein stabilizer. Instead of using glutaraldehyde vapor (Koide and Yokoyama, 1999), CLPVA was applied as a solid support due to the ability to form very homogenous films with very high quality. Redox hydrogel poly(allylamine) ferrocene was prepared by cross-linking poly(allylamine) hydrochloride with glutaraldehyde and attaching the ferrocene covalently based on established procedure by Koide and Yokoyama (1999). Amino group of cross-linked poly(allylamine) and carboxyl group of ferrocene carboxylic acid were activated using carbodiimide reagents. In this research, the effect of BSA loading also was investigated.

In the second method, ferrocene carboxylic acid and glucose oxidase were immobilized in composite sol-gel-silica (SGS) matrix containing cross-linked poly(vinyl alcohol) (CLPVA) with nafion. The membrane was prepared based on Niu and Lee (2002) but with some modifications in preparation of the sol-gel as well as CLPVA. The effect of GOD loading also was investigated for this type of membrane. Niu and Lee (2002) reported that SGS/CLPVA/nafion was an excellent matrix that can prevent the leaking of enzyme and mediators from the membrane without affecting enzyme activity and membrane stability. Besides, the hydroxyl groups in PVA may substitute for the bound water that is essential for the retention of protein tertiary structures and thus leading to activity stabilization (Niu and Lee, 2002).

In the third method, glucose oxidase and redox poly(allylamine) ferrocene was immobilized by layer-by-layer covalent attachment with the addition of nafion. The membranes was constructed by the formation of Schiff base bonds between aldehyde groups of periodate-oxidized GOD and amino groups of PAA-Fc as proposed by Zhang *et al.* (2004). PAA-Fc prepared from first method was in the form of small particles and were not suitable for layer-by-layer attachment. Poly(allylamine) ferrocene have been prepared by formation of Schiff bond between ferrocene carboxaldehyde and poly(allylamine) hydrochloride. However, in this

research, the membrane was modified by attaching nafion to increase response linearity.

Finally, for outer layer studies, the layer-by-layer membrane was coated with three types of outer membranes that can protect the enzyme layer and also extend the linear range to higher glucose concentrations. The outer membranes were cellulose acetate incorporated poly(ethylene glycol) (CA-PEG) membrane, poly(2-hydroxyethyl methacrylate) (pHEMA) and cellulose acetate incorporated poly(2-hydroxyethyl methacrylate) (CA-pHEMA). As reported by Choi *et al.* (2002), PEG was incorporated into CA membrane in order to modify the membrane hydrophilicity to give larger response. Nevertheless, in this research, CA-PEG was used to enhance the glucose linearity since the hydrophobicity of this membrane was adjustable. Besides, pHEMA was used in this research since these biocompatible hydrogels can be a potential outer membrane with the ability in limiting the glucose substrate as reported by Kermis *et al.* (2003).

Lastly, CA-pHEMA was proposed as an outer membrane since hydrophobic CA can modify the hydrophilicity of the pHEMA membrane. In addition, CA-pHEMA blended has been used by Yamashita and Endo (2006) in studying the deacetylation behaviour of binary blend of CA and various polymers. However, the biodegradable rate of the CA films was not observed for the CA films containing pHEMA. The result showed that CA-pHEMA blended can be considered stable and well-mixed and thus can be applied as a membrane. Therefore, by increasing the membrane hydrophobicity, the glucose flux can be minimized and thus increasing the linearity of glucose biosensor.

1.2 Objective

The objective of this research is to develop a ferrocene based glucose biosensor with extended linearity and minimal mediator leaking.

1.3 Scopes

To achieve the objective, the following specific scopes will be looked into:

1. Investigation of the performance of three types of immobilization methods for ferrocene based glucose biosensor especially with regard to tethering ability and sensor stability:
   - i) cross-linked poly(vinyl alcohol) (CLPVA) with the addition of bovine serum albumin (BSA) as a protein stabilizer
   - ii) sol-gel-silica (SGS) matrix containing cross-linked poly(vinyl alcohol) (CLPVA) with nafion
   - iii) layer-by-layer covalent attachment by formation of Schiff base bonds between aldehyde groups of periodate-oxidized GOD and amino groups of PAA-Fc

2. Investigation of three types of outer membranes to extend the linearity of ferrocene based glucose biosensor:
   - i) cross-linked poly(2-hydroxyethyl methacrylate) (pHEMA)
   - ii) cellulose acetate incorporated poly(2-hydroxyethyl methacrylate) (CA-pHEMA)
   - iii) cellulose acetate incorporated poly(ethylene glycol) (CA-PEG)

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